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Evaluation and Refinement of Euthanasia Methods for *Xenopus laevis*

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The most common method of euthanasia for *Xenopus* species is by immersion in tricaine methane sulfonate solution (MS222). A wide range of doses of MS222 (0.5 to 5 g/L) have been recommended, but few reports describe dose–response testing, the time to loss of consciousness, or the reliability of euthanasia. The objective of this study is to evaluate the efficacy of immersing individual and groups of frogs in MS222 at concentrations ranging from 1 to 5 g/L for euthanasia and of 3 less-common methods: intracoelomic injection of MS222, intracoelomic injection of sodium pentobarbital with phenytoin, and ventral cutaneous application of benzocaine gel. Our results indicate that immersion for at least 1 h in a 5-g/L buffered solution of MS222, intracoelomic injection of 1100 mg/kg sodium pentobarbital with sodium phenytoin (equivalent to 0.3 mL solution per frog), or ventral cutaneous application of 182 mg/kg benzocaine (equivalent to a 2 cm × 1 mm of 20% benzocaine gel) is necessary to euthanize adult *X. laevis* and ensure complete cessation of the heartbeat without recovery. These doses are considerably higher than those previously recommended for this species.

Abbreviation: MS222, tricaine methane sulfonate.

Xenopus laevis is one of the most widely used aquatic amphibians in biomedical research. With the completion of the sequencing of *X. tropicalis* genome, the use of *Xenopus* spp. in research will likely increase.[19,20] As the number of *Xenopus* used in research continues to rise, additional refinements in humane and efficient euthanasia methods must be addressed. Because *Xenopus* are fully aquatic lung breathers and exchange water, electrolytes, carbon dioxide, and oxygen through their skin,[6,7,13,16,18] typical euthanasia methods in mammals are often unsuitable for *Xenopus*. Some physical methods such as pithing are not acceptable as the sole method and can be technically challenging, leading to inconsistent unconsciousness and death in specific species.[2] In addition, amphibians are fairly tolerant to hypoxia and hypertension, rendering several euthanasia methods conditionally acceptable or unacceptable.[2] For instance, decapitation and pithing of the brain should follow pithing of the spinal cord for immediate death, according to the AVMA guidelines on euthanasia for amphibians.[2] Therefore, less technically difficult options such as anesthetic injection or immersion allow less stressful (for both personnel and frog) and more humane euthanasia. To date, *Xenopus* euthanasia methods described are largely empirical, based on clinical lore, or derived from other species.[1-2,4,10,11,29,21,23,24,31,32]

The Guide for the Care and Use of Laboratory Animals defines euthanasia as the "act of killing animals by methods that induce rapid unconsciousness and death without pain or distress."[17] The following criteria for humane euthanasia should be considered: irreversibility, reliability, a rapid time to induce unconsciousness, minimal restraint, ease and safety of administration by trained personnel, and species and age limitations.[23,29]

In most species, whether aquatic or not, chemical methods of euthanasia generally involve anesthetic overdose. Three anesthetic agents that have been used for euthanasia are benzocaine, tricaine methane sulfonate (MS222, an isomer of benzocaine), and sodium pentobarbital. Benzocaine and MS222 block the generation of action potentials by altering the properties of voltage-gated Na^+ channels.[26] Sodium pentobarbital inhibits neurotransmitter release at the synapse by binding to GABA and AMPA receptors as well as by acting directly on Ca+-dependent channels.[25] All 3 agents act directly on the central nervous system (and likely locally) to depress respiratory and cardiovascular functions, but the exact mechanism of action in *Xenopus* spp. is not well studied.

MS222 is the anesthetic typically used to euthanize fish and aquatic amphibians and is the only anesthetic approved by the FDA for anesthesia in fish. Most of the aquaculture literature regarding this agent focuses on its use in fish, with few references to other aquatic species. Careful consideration of the use of MS222 in *Xenopus* spp. is appropriate. A wide range of concentrations and routes of administration currently are recommended for the use of MS222 in amphibians: 250 to 5000 mg/L for immersion and 100 to 300 mg/kg for intracoelomic injection both with and without secondary methods of euthanasia.[1,2,4,10,11,21,23,24,29,31,32] In our clinical experience, doses of MS222 commonly used for immersion (250 to 500 mg/L) or intracoelomic injection (200 mg/L) were often ineffective in euthanizing *X. laevis* and involved prolonged time (greater than 1 h) to loss of consciousness. Therefore, we evaluated the time required to achieve complete and irreversible cessation of heart contraction after immersion of individual or a group of 5 frogs into MS222 solutions at concentrations of 1, 2, 3, and 5 mg/L. We compared the results to those from frogs euthanized by intracoelomic injection of buffered MS222 (2590 mg/kg MS222), intracoelomic injection of sodium pentobarbital with sodium phenytoin (1100 mg/kg sodium pentobarbital and 141 mg/kg sodium phenytoin), or a novel method: ventral application of 20% benzocaine gel (182 mg/kg benzocaine).

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Materials and Methods

Animals, housing, and husbandry. The *Xenopus laevis* studied were all sexually mature, adult female frogs [age, 2 to 3 y; body weight (mean ± 1 SD), 109 ± 20.6 g; snout-to-vent length, 10.7 ± 0.15 cm] previously scheduled for euthanasia. All frogs were housed in dark-green, opaque, bathtub-style tanks (width, 4 ft; length, 6 ft; height, 4 ft), and filled with 300 L dechloraminated potable water. The average daily census was approximately 150 frogs per tank (a minimum of 2 L water per frog following recommendations of the *National Academy of Sciences* [29]) with approximately 15 frogs removed each month for egg harvest. All frogs were fed a commercial pelletted diet (*Xenopus* Brittle, NASCO, Madison, WI) 3 times each week, 3 h before automated 100% draining and refilling of the tank water (temperature, 16 to 21 °C). Water quality was monitored approximately once every 6 to 8 mo. Water-quality parameters were tested (Voluette Analytical Standards, Hatch Company, Loveland, CO) and maintained within the ranges considered safe for aquatic amphibians: pH, 7.0 to 8.5; total chlorine, less than 0.01 mg/L; chloramines, less than 0.01 mg/L; ammonia, less than 0.25 mg/L; nitrite, less than 0.20 mg/L; nitrate, 0.00 to 50.0 mg/L; copper, less than 0.02 g/L; water fecal coliform counts, less than 2000 per 100 mL; conductivity, 300 to 1000 μΩ; and dissolved oxygen, 8.00 to 9.00 mg/L. Room conditions included a 12:12-h light:dark cycle and ambient temperature of 23 to 25 °C. Frogs were provided environmental enrichment in the form of acrylonitrile–butadiene–styrene pipes (inner diameter, 4 in.; Plastic Pipe Fittings Association, Glen Ellyn, IL).[30]

Immersion in MS222. A total of 160 frogs were divided into groups of 20 each to test each dose level (1, 2, 3, and 5 g/kg) for individual and group immersions. Solutions of MS222 for immersion were prepared by mixing MS222 powder with fresh water from the housing tank. The solution was buffered with pharmaceutical-grade sodium bicarbonate to a pH of 7 to 7.5, and temperature was maintained between 16 and 21 °C. For each concentration level, 20 frogs were immersed either individually or in groups of 5 frogs. To this end, 10 L of solution was divided among 5 polycarbonate cages (cage size, 11 × 6.5 × 4.5 in.) for immersion of individual frogs or poured into a 20-L bucket for group immersion of 5 frogs. The same source of MS222 (new bottle) was used throughout the study, and all solutions were prepared on the same day with water from the same tank. For all group immersions, all animals were left in the immersion solution for 1 h. A histologic review was performed on 12 frogs, 3 frogs from each concentration level of the group immersions.

Intracoelomic injection of MS222. Each of 20 frogs (body weight, 112 ± 20 g; snout-to-vent length, 10.7 ± 0.67 cm) was injected intracoelomically with 2590 mg/kg buffered MS222 [reaching the water solubility of MS 222 (1250 mg/mL) at 20 °C,[28] because 2 mL were prepared for each frog] and placed in water until deep anesthesia was confirmed.

Intracoelomic injection of sodium pentobarbital with sodium phenytoin. Each of 20 frogs was injected intracoelomically with 1100 mg/kg sodium pentobarbital and 141 mg/kg sodium phenytoin (0.3 mL of solution per frog) and placed in water until deep anesthesia was confirmed.

Application of 20% benzocaine gel. Each of 20 frogs received 182 mg/kg benzocaine through application of a 2 cm × 1 mm strip (wet weight, 100 mg) of 20% benzocaine gel on its ventral abdomen (Figure 1). The gel was applied directly, without any preparation of the skin, from the tip of the tube. Personnel wearing wet gloves restrained frogs manually during gel application, after which each frog was returned to a wet bucket without water until deep anesthesia was confirmed.

Experimental methods for all groups. All experiments were performed in accordance with protocols approved by our institutional animal care and use committee. Preliminary studies were conducted on small groups of frogs (n = 5 per group) to determine the appropriate doses ranges (for example, doses that provided deep anesthesia for at least 1 h) for MS222 (Finquel, Argent Chemical Laboratories, Redmond, WA) administered by immersion or intracoelomic injection, intracoelomic injection of sodium pentobarbital and phenytoin (Beuthanasia, Schering Plough Animal Health, Union, NJ), and ventral cutaneous application of benzocaine (Anbesol, Wyeth Consumer Healthcare, Richmond, VA).

Twenty frogs were randomly assigned to each experimental group and dose. Immediately upon immersion, intracoelomic injection or gel application, frogs were monitored continuously for withdrawal and righting reflexes disappearance confirming deep anesthesia. Individuals were then removed from their original bucket (after 1 h of immersion in the case of MS222 treatments or after deep anesthesia was confirmed for the other methods) and placed on their backs on a moistened plastic board to allow for observation of heart contractions at the ventral midline beneath the sternum (hearts could be seen beating readily beneath the skin). The withdrawal reflex was tested and visual observation of the frog's heartbeat was performed every 15 min for the duration of the study and recorded at 1 h, 3 h and 5 h after drug administration or removal from immersion. Visualization of the heart by opening the coelomic cavity was performed to confirm cessation of the heart contractions once externally imperceptible (frogs deeply anesthetized). Death was defined as the complete cessation of the heart contractions. When the heart did not stop after 5 h, a secondary method of euthanasia (either physical (pithing) or chemical with intracardiac injection of sodium pentobarbital with sodium phenytoin) was performed after deep anesthesia was ensured (see Figures 2 to 5). After 5 h, all frogs without a heartbeat from all groups were placed in a 4 °C cooler. Hearts were rechecked 15 h later, including 1 h left outside the cooler, to confirm the hearts did not start beating again.

No statistical analysis was performed. We calculated the percentage of animals still showing heart contraction at different time points for each method and each dose and compared the methods based on these results. In our opinion 100% confirmed death within a minimal time period is the only result acceptable.

Results

Immersion in MS222. ***Immersion of individual frogs.*** Deep anesthesia of individual *Xenopus* frogs was achieved at all 4 concentrations of MS222 in less than 4 min on average (Figure 2). However, immersion for 1 h led to recovery of mobility without cessation of the heartbeat in 6 of the 20 frogs in the 1-g/L group and in none of the 20 in the 2-g/L group. In addition, 20 h after removal from immersion, 2 frogs in the 1000-mg/L and 1 frog in the 2000-mg/L solution had a heartbeat (data not shown). Immersion in MS222 at 3 g/L resulted in complete and irreversible euthanasia of all frogs by 5 h. Immersion for 1 h in 5-g/L MS222 resulted in the death of all frogs by 3 h after removal from the anesthetic solution.

Group immersion in MS222. Deep anesthesia of all 5 frogs in each group was reached within 4 min at all MS222 concentrations (Figure 3). However, at 1 g/L, only 2 of the 20 frogs tested had no heartbeat after 5 h; 5 frogs were actively moving at 3 h and another 2 at 5 h after removal from the anesthetic solution. These frogs were euthanized by a secondary method

previously described. The 2-g/L solution had similar results to the 1-g/L solution (Figure 3). The 3-g/L solution effectively euthanized (that is, loss of mobility and complete cessation of the heartbeat) all frogs in 5 h .The most concentrated solution (5 g/L) euthanized all frogs with complete cessation of the heart in 3 h. At 1 h after removal from the 5-g/L solution, complete cardiac arrest had occurred in 2 of the 20 hearts, and another 7 hearts showed only contraction of the atrium.

Intracoelomic injection of MS222. At the highest possible dose (2590 mg/kg), MS222 injected intracoelomically did not result in euthanasia (that is, cessation of heart contraction) of *Xenopus* frogs within 5 h after injection. Of the 20 frogs in the group, 6 recovered mobility between 1.5 h and 3 h after injection. All frogs in this group were euthanized by a secondary method.

Intracoelomic injection of sodium pentobarbital and sodium phenytoin. Combined dosage with 1100 mg/kg sodium pentobarbital and 141 mg/kg sodium phenytoin (0.3 mL of solution per frog) led to complete cardiac arrest within 3 h after injection without recovery of any frogs (17 of the 20 frogs were dead 1 h after injection; Figure 4).

Ventral cutaneous application of 20% benzocaine gel. Ventral cutaneous application of 2 cm × 1 mm strips (Figure 1) of 20% benzocaine gel (equivalent to 182 mg/kg benzocaine; wet weight of 100 mg) euthanized 100% of the frogs within 5 h. Notably, 18 of the 20 frogs tested were dead after 3 h. Righting and withdrawal reflexes subsided in less than 7 min after application of the gel. The frogs' skin remained well hydrated at all times during the experiment. Sloughing or other signs of injury to the skin and difficulty breathing were not observed.

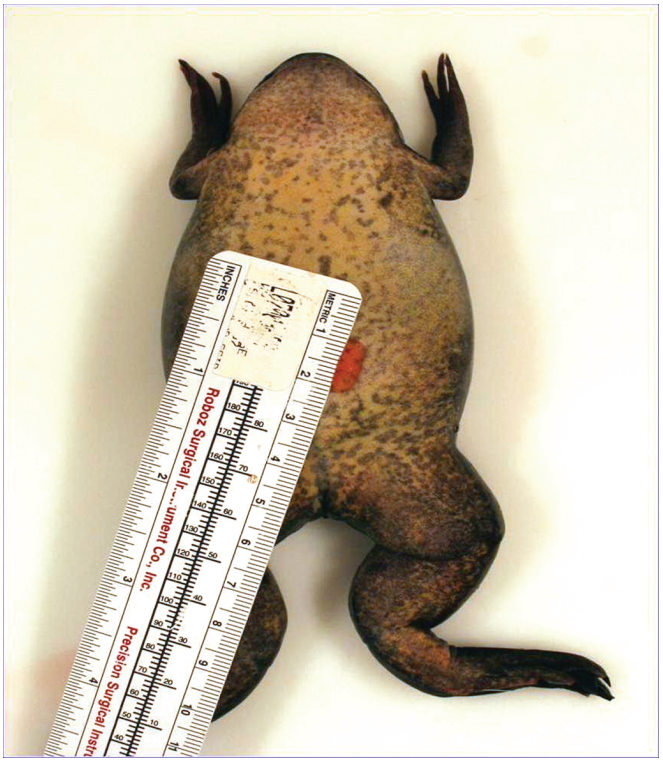

Figure 1. An adult female South African clawed frog (*Xenopus laevis*) with 1 cm × 1 mm ventral application of 20% benzocaine gel.

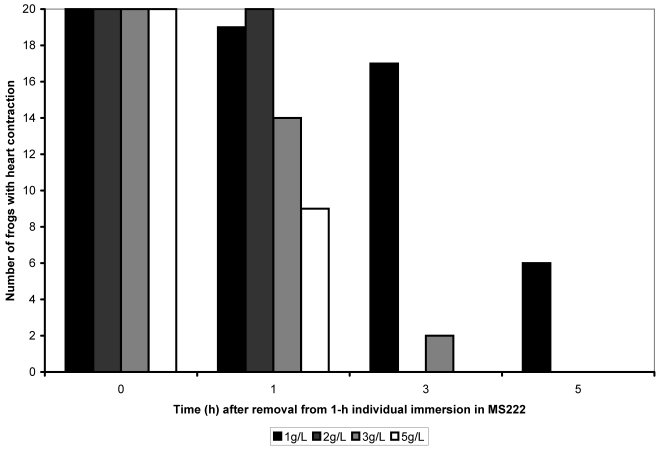

Figure 2. Numbers of *Xenopus* frogs with heart contraction at 0, 1, 3, and 5 h after individual immersion in 1-, 2-, 3-, and 5-g/L solutions of MS222.

Discussion

The results of our comparative testing indicate that intracoelomic injection of sodium pentobarbital with sodium phenytoin and immersion for 1 h in MS222 solution at 5 g/L are the most effective and rapid methods for euthanizing, respectively, small and large numbers of frogs. The novel euthanasia method, ventral cutaneous application of benzocaine gel (182 mg/kg), proved to be a reliable method of euthanasia, although slower than pentobarbital and immersion in MS222 at 5 g/L. These findings reinforce our clinical observations: there is great variability in the efficacy of MS222 administered by immersion or intracoelomic injection and in intracoelomic coinjection of sodium pentobarbital and sodium phenytoin (intracoelomic injection) as methods of euthanasia for *Xenopus* spp. In addition, the reported (lower) doses of these agents are not appropriate for *Xenopus* frogs; the doses we found to be effective are considerably higher than those previously published. [2,4,10,11,31,32]

Many factors likely affect the variability of MS222 on *Xenopus*: metabolic rate, respiration–skin exchange ratio, and water absorption rate are linked directly to water temperature, pH, and composition; age and sex of the animal; and season.[6,7,13] Lower water temperature and greater age lower metabolic rates and prolong the time to achieve anesthesia and euthanasia.[6,13,14]

A disadvantage of using MS222 for euthanasia is its potential toxicity to humans. If inhaled or absorbed chronically through the skin, MS222 induces reversible retinal toxicity.[5] MS222 must be handled with care, preferably by trained personnel, who should prepare the solution under a fume hood and wear nitrile gloves, mask, and eye protection. MS222 powder is susceptible to degradation over time and must be stored in a lightproof container, preferably in a freezer. The shelf-life of MS222 powder can be at least a year when stored under these conditions.[3] In addition, we found that MS222 solutions remain effective for several days as long as they are maintained at constant temperature and in lightproof containers. Unbuffered solutions of MS222 are acidic and will irritate the skin and coelomic cavity of the animals; therefore solutions must be buffered before each use. Because MS222 is absorbed most readily at neutral pH,[22] buffering the solution will both improve effectiveness and reduce the likelihood of pain.

The use of 20% benzocaine gel as a euthanasia agent for *Xenopus laevis* has not previously been described. We found this method to be suitable and convenient, particularly for field experiments or when only a few frogs are euthanized at a time. Benzocaine gel has been evaluated as an anesthetic for administration by immersion[8] or as a euthanasia agent by ventral cutaneous application in other amphibian species.[4,9] Although a rare side effect, benzocaine can cause methemoglobinemia in several laboratory species when used topically or systemi-

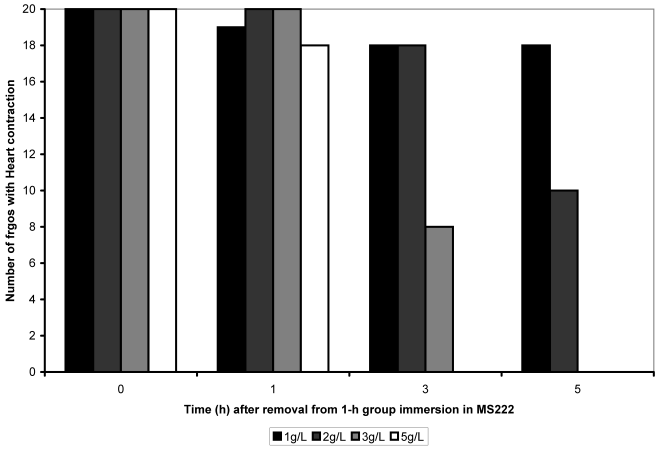

Figure 3. Numbers of *Xenopus* frogs with heart contraction at 0, 1, 3, and 5 h after group (n = 5) immersion in 1-, 2-, 3-, and 5-g/L solutions of MS222.

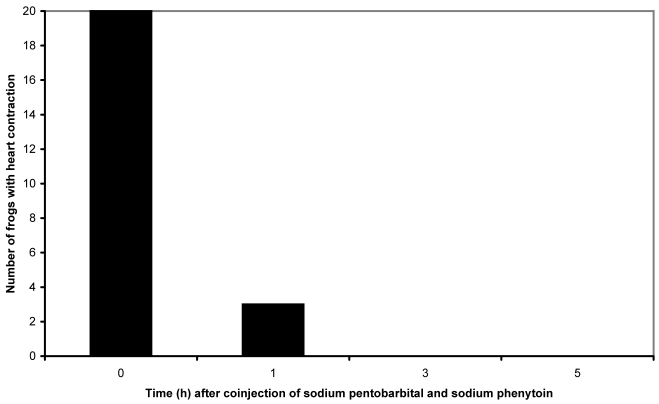

Figure 4. Numbers of *Xenopus* frogs with heart contraction at 0, 1, 3, and 5 h after intracoelomic coinjection of sodium pentobarbital (1100 mg/kg) and phenytoin (141 mg/kg) solution.

cally.[12] Similarly, humans are susceptible to benzocaine,[15,27] but preparations of benzocaine gel available without prescription are relatively safe, and users can easily protect themselves by wearing nitrile gloves.

Combined intracoelomic injection of sodium pentobarbital (1100 mg/kg) and sodium phenytoin is a rapid and effective method for euthanasia of *Xenopus* frogs. However regulatory requirements (pentobarbital is a Schedule III controlled drug) might be prohibitive. In addition, this method can be expensive and time-consuming when numerous frogs must be euthanized. For researchers using *Xenopus* spp., the potential to damage tissues is always of concern. For instance, combined administration of sodium pentobarbital and sodium phenytoin is known to induce histologic alterations of tissues when injected intracoelomically.[31] However, the histologic review of tissues from 12 frogs euthanized by MS222 immersion in our study (data not shown) did not show any evidence of tissue damage.

In most cases, the heart was still beating when the coelomic cavity was opened, even though none of the animals demonstrated withdrawal and righting reflexes or had externally visible heart contraction. Therefore we recommend maintaining frogs in 5-g/L MS222 solution for at least 1 h. If the MS222 solution must be used at a concentration below 5 g/L or for a shorter period of time, a secondary method of euthanasia should be used. Quick freezing, double pithing, removing the heart, or

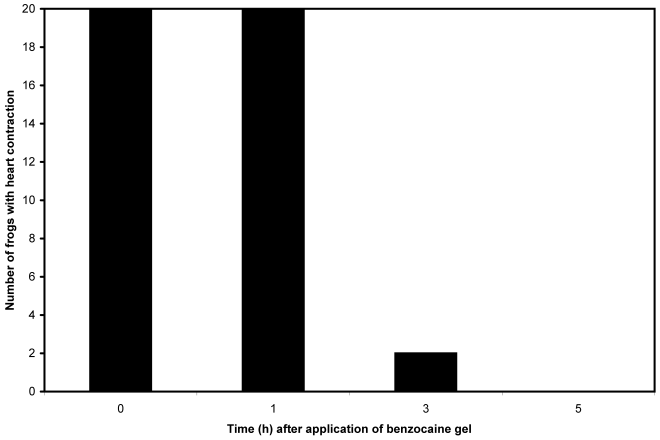

Figure 5. Numbers of *Xenopus* with heart contraction at 0, 1, 3, 5 h after ventral cutaneous application of 182 mg/kg benzocaine (that is, 2 cm × 1 mm strip of 20% benzocaine gel).

decapitation after deep anesthesia are all acceptable methods cited in the AVMA guidelines.[2]

The findings reported here suggest that doses of MS222 and sodium pentobarbital with sodium phenytoin traditionally recommended for euthanasia of amphibians are too low for reliable euthanasia of adult, female *Xenopus laevis* frogs.[2,4] Given the water quality, housing and husbandry conditions, and signalment of the animals used in the current study, intracoelomic coinjection of sodium pentobarbital (1100 mg/kg) and sodium phenytoin, ventral cutaneous application of 20% benzocaine gel (total dose, 182 mg/kg), and 1-h immersion in 5 g/L MS222 are practical, quick, and effective methods for euthanasia of *Xenopus laevis.* We recommend the first 2 methods for euthanasia of individual animals and for use in the field, and immersion in MS222 for euthanasia of large numbers of adult *Xenopus laevis*.

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